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**Pharmacological potential of novel agonists for FFAR4 on islet and enteroendocrine cell function and glucose homeostasis**

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**Short Title:** Selective FFAR4 agonists and beta cell function

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## **Abstract**

**Background:** To investigate the metabolic effects of FFAR4-selective agonists on islet and enteroendocrine cell hormone release and the combined therapeutic effectiveness with DPP-IV inhibitors.

**Methods:** Insulinotropic activity and specificity of FFAR4 agonists were determined in clonal pancreatic BRIN-BD11 cells. Expression of FFAR4 was assessed by qPCR and western blotting following agonist treatment in BRIN-BD11 cells and by immunohistochemistry in mouse islets. Acute *in-vivo* effects of agonists was investigated after intraperitoneal (i.p.) or oral administration in lean and HFF-obese diabetic mice.

**Results:** GSK137647 ( $10^{-11}$ - $10^{-4}$  M) and Compound-A ( $10^{-10}$ - $10^{-4}$  M) stimulated insulin secretion at 5.6mM ( $p<0.05$ - $p<0.001$ ) and 16.7mM ( $p<0.05$ - $p<0.001$ ) glucose in BRIN-BD11 cells, with no cytotoxicity effects as assessed by MTT. FFAR4 antagonist (AH-7614) abolished the insulinotropic effect of GSK137647 ( $p<0.05$ - $p<0.001$ ), whilst FFAR1 antagonist (GW1100) had no effect. Incubation of BRIN-BD11 cells with GSK137647 and Compound-A increased FFAR4 ( $p<0.01$ ) gene expression at 16.7 mM glucose, with a corresponding increase in FFAR4 ( $p<0.01$ ) protein concentrations. FFAR4 upregulation was attenuated under normoglycaemic conditions. Immunohistochemistry demonstrated co-localisation of FFAR4 and insulin in mouse islets. Orally administered GSK137647 or Compound-A (0.1  $\mu$ mol/kgBW) monotherapy and combinational therapy with Sitagliptin improved glucose tolerance ( $p<0.001$ ), increased plasma insulin ( $p<0.001$ ), GLP-1 ( $p<0.05$ ), GIP ( $p<0.05$ ), decreased DPP-IV activity ( $p<0.01$ - $p<0.001$ ) and induced satiety ( $p<0.001$ ) in HFF mice.

**Conclusions:** Specific FFAR4 agonism improves glucose tolerance through insulin and incretin secretion, with enhanced DPP-IV inhibition in combination with Sitagliptin.

**General significance:** These findings have for the first time demonstrated that selective FFAR4 activation regulates both islet and enteroendocrine cell function with agonist combinational therapy, presenting a promising strategy for the treatment of type-2-diabetes.

**Keywords:** FFAR4, specificity, insulin, incretin, DPP-IV inhibition, combinational therapy

## **1 Introduction:**

FFAR4 (GPR120) is a rhodopsin-like G-protein coupled receptor that is activated by unsaturated fatty acids (C16-22) and long chain saturated fatty acids (C14-18) [1-2]. The human FFAR4 gene is encoded on chromosome 10.q23.3 [3]. With respect to tissue distribution, FFAR4 is extensively expressed in peripheral tissues, intestines, lungs, spleen and pro-inflammatory macrophages [4]. Furthermore, recent studies have demonstrated that FFAR4 is abundantly expressed in the pancreatic islet, with further analysis demonstrating its expression in clonal pancreatic  $\beta$ -cell lines, including MIN6, RINm5f and INS-1E [5-7].

Previously considered as orphan receptors, recent studies have shown FFAR1 (GPR40), FFAR2 (GPR43), FFAR3 (GPR41), FFAR4 (GPR120) and GPR84 to be activated by free fatty acid (FFA) molecules [3, 4, 8]. FFAR3 and FFAR2 exhibit specificity towards short chain fatty acids, GPR84 is activated by medium chain fatty acids, whereas FFAR1 and FFAR4 are activated by long chain fatty acids [3, 4]. Furthermore, FFAR4 and FFAR1 share 10% sequence homology and can be activated by similar endogenous ligands (Omega-3-fatty acids), which warrants the utilisation of receptor specific agonists to evaluate the therapeutic potential of FFAR4 [2, 8].

FFAR4 has been hypothesised to act as a lipid sensor in the body, and has been proven to have involvement in the regulation of inflammation, adipogenesis, and glucose homeostasis [1, 7, 9]. Interestingly, it has been reported that a mutation in the FFAR4 gene (R270H) is linked with the development of obesity. The p.R270H variant impairs the signalling response of FFAR4 upon FFA binding, with subsequent defects observed to intracellular calcium mobilisation and GLP-1 secretion in intestinal cells [10, 11]. Further studies have demonstrated that FFAR4 knockdown with siRNA impaired the anti-apoptotic effects of omega-3 fatty acids in serum-starved STC-1 cells. Thus, indicating the potential proliferative and anti-apoptotic effects of FFAR4 in pancreatic beta cells [12].

Numerous studies have identified the involvement of FFAR4 in the gastrointestinal (GI) tract, including the mediation of glucagon-like peptide-1 (GLP-1), gastric inhibitory polypeptide (GIP) and cholecystokinin (CCK) secretion from intestinal L-cells, K-cells and I-cells, with high FFAR4 expression observed in the intestinal STC-1 and GLUTag cell lines [1, 12, 13]. FFAR4 activation has been shown to mediate GLP-1 secretion when tested with its endogenous agonist  $\alpha$ -linolenic acid [1, 14, 15]. However, other studies suggest that FFAR4 has no role in GLP-1 release [16]. Previous findings have shown FFAR4 to mediate insulin-sensitising and anti-inflammatory properties in peripheral tissues [9].

The expression and biological function of FFAR4 in the intestinal tract has been heavily documented, however the role of FFAR4 in pancreatic beta cell function was not investigated until recently [7]. A number of FFAR4 agonists were demonstrated to have regulatory role in glucose dependent insulin secretion in mouse islets, including endogenous docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), alpha-linolenic acid (ALA) and synthetic GW-9508 [7, 8]. In addition, these agonists demonstrated insulinotropic and glucose lowering properties in *in-vivo* [7]. However, the selectivity of endogenous FFAR4 agonists (ALA, DHA, EPA)

remains uncertain as activation of FFAR1 may contribute to the effects observed, whilst synthetic GW9808 has been shown to exhibit 100-fold greater potency towards FFAR1 over FFAR4 [17, 18].

Upon activation, FFAR4 primarily couples to G $\alpha$ <sub>q</sub>, which stimulates an array of secondary messenger signalling pathways through phospholipase C (PLC), including intracellular calcium and mitogen-activated protein kinases [4, 7]. The mechanism of FFAR4 mediated insulin secretion from the pancreatic beta cell is not conclusive; however, studies have shown a range of FFAR4 agonists to induce intercellular calcium release, indicating the potential involvement of inositol trisphosphate on intracellular calcium stores through PLC $\beta$  signalling [7]. FFAR4 activation with ALA and DHA leads to the rapid and transient phosphorylation of the receptor of HEK293 cells [19]. Although FFAR4 has been shown to act predominately through PKC signalling, DHA has also shown to activate G-protein coupled receptor kinase (GPK6) upon FFAR4 phosphorylation, with Thr(347), Ser (350), and Ser(357) shown to be major phosphorylation sites in the C-terminal tail of FFAR4 [19].

Recently, Oh *et al.*, have reported an orally available, selective, high affinity, small FFAR4 agonist (Compound A) that exhibits a range of anti-diabetic effects [20]. Oral administration of Compound A improved glucose tolerance, insulin sensitivity and exerted anti-inflammatory effects on macrophages in high fat fed obese mice [20]. Sparks *et al.*, recently identified a potent FFAR4 agonist GSK137647 [21] and preliminary *in-vitro* analysis has demonstrated that GSK137647 augmented insulin secretion in MIN6 cells, with a modest increase in GLP-1 secretion from the NCI-H716 intestinal cell line [21, 22]. *In-vivo* findings showed that GSK137647 induced GLP-1 release by mouse circumvallate papillae [22]. The highly selective properties of this agonist suggested that it was suitable to evaluate FFAR4 activation in pancreatic beta cells.

Due to the regulatory role of FFAR4 activation on insulin and incretin secretion [1, 7, 14], a promising approach using selective FFAR4 agonists combined with a dipeptidyl peptidase-4 (DPP-IV) inhibitor may offer therapeutic potential [23, 24]. The present study has assessed the effect of potent DPP-IV inhibitor (Sitagliptin) in combination with Compound A and GSK137647 on glucose tolerance and insulin secretion in high fat fed (HFF)-induced diabetic mice. This research aims to investigate the acute metabolic effects and of FFAR4 agonist monotherapy and combinational therapy on islet and enteroendocrine cell function, using pancreatic cells and diabetic mice.

## **2 Materials and methods:**

### **2.1 Materials:**

FFAR4 agonists Compound A and GSK137647 were purchased from Cayman Chemicals (Michigan, USA) and Tocris (Bristol, UK) respectively. Sitagliptin phosphate monohydrate was obtained from Apexbio Technology LLC (Texas, USA). Thiazolyl blue tetrazolium bromide (MTT) was received from Sigma (Poole, UK). Rabbit anti-GPR120 polyclonal IgG antibody (H-155) was purchased from Santa Cruz biotechnology (Santa Cruz, CA, USA) and guinea pig anti-insulin from Abcam (Cambridge, UK).

### **2.2 Insulin secretion:**

Generation and characterization of the insulin-secreting BRIN-BD11 cells were outlined previously [25]. BRIN-BD11 cells were cultured with RPMI-1640 media (11.1 mM glucose) containing antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin) and 10% foetal calf serum at 37°C in 95% air and 5% carbon dioxide. For acute insulin secretion studies, cells were detached using trypsin/EDTA and incubated overnight in 24-well plates with 150,000 cells per

well. Cells were then pre-incubated for 40 min at 1.1 mmol/l glucose in Krebs buffer (comprising 4.7 mmol/l KCL, 115 mmol/l NaCl, 1.28 mmol/l CaCl<sub>2</sub>, 10 mmol/l NaHCO<sub>3</sub>, 5 g/l bovine serum albumin, 1.2 mmol/l KH<sub>2</sub>PO<sub>4</sub>, 1.2 mmol/l MgSO<sub>4</sub>·7H<sub>2</sub>O, pH 7.4). Test incubations were then performed at 37°C for 20 min. Supernatants were removed, then frozen at -20°C until determination of insulin by radioimmunoassay [26]. All FFAR4 ligands (1 mg/ml) were dissolved in 50% DMSO prior to preparation in Krebs buffer. Compound A and GSK137647 at 10<sup>-12</sup>-10<sup>-4</sup> mol/l were tested at 5.6 and 16.7 mmol/l glucose. To determine the selectivity of the agonists towards FFAR4, the FFAR1 antagonist GW1100 (10<sup>-5</sup> mol/l), and FFAR4 antagonist AH-7614 (10<sup>-5</sup> mol/l) were utilised to antagonise the respective receptors. Receptor antagonists were co-incubated with Compound A and GSK137647 on BRIN-BD11 cells, with insulin secretory responses determined.

### **2.3 Cytotoxicity assessment by tetrazolium (MTT):**

BRIN-BD11 cells were cultured overnight in 96-well plates. Incubations were performed as described for insulin secretory analysis. Test solutions were decanted, then 1mg/ml of working MTT solution was added and incubated for 2 h at 37°C. MTT solution was removed and replaced with 200 µl of DMSO. The plate was placed on an orbital shaker for 5 min to mix the formazan into the solvent. The optical density of each well was recorded at 560 nm with the background absorbance at 670 nm removed. The resultant optical density was proportional to the viable cell quantity.

### **2.4 Acute effects of FFAR4 agonists *in-vivo*:**

All animal experiments were carried out in accordance with the UK Animal (Scientific Procedures) Act 1986. Male lean and HFF Swiss TO mice (Harlan UK, 30-34 weeks old, 55–67 g) were individually housed in an air-conditioned room at 22 ± 2°C with a 12-h light: 12-h dark cycle. Drinking water was supplied ad libitum. Animals were maintained on a high fat



diet (45% fat, 20% protein, 35% carbohydrate; percent of total energy 26.15 kJ/g; Dietex International Ltd., Witham, UK) from 8 weeks of age for a total of 150 days to evoke dietary-induced obesity-diabetes (DIO). Another group of mice was maintained on standard rodent diet (10% fat, 30% protein, 60% carbohydrate; percent of total energy 12.99 kJ/g, Trouw Nutrition, Cheshire, UK) and used as a model of normal controls. Similar high-fat diets, containing a large percentage of energy from fat, are used routinely in obesity-diabetes research [27-29]. Mice (n=6, fasted 18 h) received an oral or IP administration of glucose alone (18 mmol/kg body weight) or in combination with FFAR4 agonists (0.1  $\mu$ mol/kg body weight). All FFAR4 ligands (1 mg/ml) were dissolved in 50% DMSO prior to preparation in saline. Blood samples were obtained by a cut from the tip of the tail vein of conscious mice at the times indicated in Figures, and centrifuged at  $16,060 \times g$  for 3 min at 4°C. Plasma glucose was measured using an automated glucose oxidase procedure with a Beckman glucose analyser (Beckman-Coulter, High Wycombe, UK) and insulin determined by radioimmunoassay [26]. Intestinal hormone secretion was assessed using ELISA; total GLP-1 (Millipore) and total GIP (Millipore). DPP-IV activity was evaluated by Gly-Pro-AMC cleavage [30]. In a second series of experiments, 18 h fasted normal mice were used to assess the effects of agonist treatment on food intake. Mice received oral administration of saline alone (0.9% (w/v) NaCl) or in combination with agonist (0.1  $\mu$ mol/kg body weight) and food intake measured at 30 min intervals.

## **2.5 Gene expression analysis by qPCR:**

mRNA was extracted from clonal pancreatic BRIN-BD11 cells following exposure to agonist treatment, using an RNeasy Mini kit adhering to manufacturer's protocol (Qiagen, UK). Isolated mRNA (3  $\mu$ g) was converted to cDNA using SuperScript II Reverse Transcriptase. Amplification parameters were set at 95°C for denaturation, 58°C for primer annealing and 72°C for elongation for a total of 40 cycles, followed with melting curve analysis, with

temperature range set at 60°C to 90°C. Values were analysed using the Livak method and normalised to GAPDH expression.

## **2.6 FFAR4 protein concentrations using western blotting:**

BRIN-BD11 cells were seeded at a density of 1,000,000 cells per well in 6-well plates and allowed to attach overnight. After 4 h exposure to 10<sup>-4</sup>M GSK137647 and Compound A, total protein was extracted at 4°C for 10 min using RIPA buffer containing 150 mM NaCl, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris HCl, pH 7.6 and protease inhibitor cocktail (Sigma, UK). Total protein concentration was determined using Bradford reagent (Sigma, UK). Equal amounts of protein were prepared in aliquots with Laemmli buffer (1 µg/µl), then boiled at 95°C for 10 min. Samples (25 µg per well) were loaded onto pre-cast gels (NUPAGE 4–12% Bis–Tris gels, Invitrogen, UK) and subjected to SDS-PAGE (70 V, 90 min). After transfer to nitrocellulose membrane for 16 h at 90 mA, membranes were blocked with 5% skimmed milk and probed with rabbit anti-FFAR4 (1:150) (Santa Cruz, US)/mouse anti-β-actin (1:2500) (Cell signalling, US). Membranes were probed with ECL horseradish peroxidase donkey anti-rabbit IgG/ECL horseradish peroxidase sheep anti-mouse IgG (1:10000) (GE Healthcare, UK) and detected using Luminata Forte HRP substrate (Millipore, UK), with images captured using the G:BOX Chemi XX9 imager (Syngene, UK). Data were normalised to β-actin and presented relative to untreated control.

## **2.7 Tissue distribution of FFAR4 by immunohistochemistry:**

Pancreatic tissue from lean and HFF NIH Swiss mice was excised and cut at 8µm using a microtome. Sections were placed on slides and dried for 2 h on a hot plate at 37°C. After incubation, wax was removed and tissue re-hydrated in ethanol (100%), ethanol (95%), ethanol (80%) and distilled water for 5 min each. Slides were incubated in 50 mM sodium citrate for 20 min at 90°C for antigen retrieval. BSA (2.5%) was added to each slide (200 µl) for 45 min.

Primary antibodies (200 µl) at optimal dilutions (FFAR4 1:100, Insulin 1:300) were added and incubated at 4°C overnight. Slides were washed, followed by secondary antibody (1:400) incubation at 37°C for 45 min. The slides were washed, then DAPI (0.1 µg/ml) added and incubated at 37°C for 15 min. Slides were washed, then mounted.

## 2.8 Statistics

All data was analysed with Prism (v.5.0, GraphPad Software Inc. CA, USA) and expressed as mean ± S.E.M. All *in-vivo* glucose tolerance test data (glucose, insulin, GLP-1, GIP and DPP-IV activity) were analysed using two-way analysis of variance (ANOVA) followed by the Bonferroni *post-hoc* test. Area under the curve (AUC) was calculated using trapezoidal rule with baseline correction. All other data including AUC were analysed using Student's *t*-test (non-parametric, with two-tailed P values and 95% confidence interval).  $p < 0.05$  was considered to be statistically significant.

## 3 Results:

### 3.1 Determination of FFAR4 agonist selectivity on insulin secretion.

The insulinotropic response and specificity of the novel synthetic FFAR4 agonists (Compound A, GSK137647) at  $10^{-12}$ - $10^{-4}$  mol/l were assessed using clonal pancreatic BRIN-BD11 cells. At 5.6 mM glucose, Compound A at  $10^{-10}$ - $10^{-4}$  mol/l augmented insulin secretion by 1.2- to 1.9-fold ( $p < 0.05$ - $p < 0.001$ ), with a half maximal effective concentration ( $EC_{50}$ ) of  $2.9 \times 10^{-7}$  mol/l, while GSK137647 was more potent ( $EC_{50}$  of  $2.2 \times 10^{-7}$  mol/l) with a 1.5- to 2.1-fold increase at  $10^{-8}$ - $10^{-4}$  mol/l ( $p < 0.05$ - $p < 0.001$ ) (Figure 1 A, B). At 16.7 mM glucose, both agonists exhibited enhanced insulinotropic activity. Compound A at  $10^{-7}$ - $10^{-4}$  mol/l augmented insulin secretion from 1.8- to 2.4 fold ( $p < 0.05$ - $p < 0.001$ ) and GSK137647 at  $10^{-11}$ - $10^{-4}$  mol/l

enhanced insulin secretion by 1.4- to 2.8-fold ( $p<0.05$ - $p<0.001$ ), with  $EC_{50}$  values of  $3.0 \times 10^{-8}$  mol/l (Compound A) and  $1.2 \times 10^{-10}$  mol/l (GSK137647) (Figure 1 C, D). Neither agonist affected cell viability when assessed by MTT (Figure 1).

To investigate the selectivity of the agonists, both Compound A and GSK137647 were co-incubated with selective FFAR1 and FFAR4 antagonists. The insulin secretory response of Compound A and GSK137647 was not influenced by incubation with the FFAR1 antagonist GW1100 ( $10^{-5}$  mol/l), with similar insulintropic responses being observed in the presence and in the absence of GW1100 (Figure 1). In contrast, the FFAR4 antagonist AH-7614 ( $10^{-5}$  mol/l) significantly impaired the insulintropic responses of Compound A and GSK137647. At 5.6 mM glucose in the presence of the FFAR4 antagonist, Compound A ( $10^{-6}$ - $10^{-4}$  mol/l) augmented insulin secretion by 1.25- to 1.3-fold ( $p<0.05$ ), corresponding to a 65% decrease in insulin output compared with control. Furthermore, the insulintropic effect of GSK137647 was abolished in the presence of the FFAR4 antagonist (Figure 1 A, B). At 16.7 mM glucose in the presence of the FFAR4 antagonist, Compound A at  $10^{-5}$ - $10^{-4}$  mol/l increased insulin secretion by 1.7- to 1.8-fold ( $p<0.05$ ), corresponding to a 65% reduction compared to agonist alone. GSK137647 at  $10^{-7}$ - $10^{-4}$  mol/l only augmented insulin secretion by 1.7- to 2.1 fold in the presence of the antagonist ( $p<0.01$ ) (Figure 1 C, D), reflecting a 40% decrease in insulintropic action.

### **3.2 Expression of FFAR4 in high fat fed pancreatic tissue and BRIN-BD11 cells:**

Immunohistochemistry revealed high expression and areas of co-localisation of FFAR4 and insulin in pancreatic islets from lean and HFF mice (Figure 2 A-H). The pancreatic BRIN-BD11 cell line was used to confirm FFAR4 gene expression in pancreatic beta cells. At 5.6 mM glucose, FFAR4 agonists Compound A ( $p<0.05$ ) and GSK137647 ( $p<0.05$ ) downregulated FFAR4 receptor mRNA expression in BRIN-BD11 cells (Figure 2 I). When

exposed to 16.7 mM glucose, FFAR4 agonists (Compound A ( $p<0.01$ ) and GSK137647 ( $p<0.01$ ) increased FFAR4 mRNA expression (Figure 2 J). Western blotting was conducted to determine complimentary FFAR4 protein concentrations after agonist treatment. GSK137647 upregulated FFAR4 protein by 1.9 fold ( $p<0.05$ ) in hyperglycaemic conditions, whilst Compound A had no significant effect (Figure 2 L). FFAR4 protein concentrations were not altered by either agonist under normoglycaemic (5.6 mM glucose) conditions (Figure 2 K).

### 3.3 Acute effects of FFAR4 agonists on glucose tolerance and insulin secretion *in-vivo*

An oral glucose tolerance test (OGTT) was performed to assess the anti-diabetic activity of Compound A and GSK137647 in fasted HFF mice. Compound A and GSK137647 were assessed alone or in combination with the DPP-IV inhibitor (Sitagliptin). FFAR4 antagonist AH-7614 was utilised to determine agonist specificity *in-vivo*. Oral administration of Compound A and GSK13647 improved glucose tolerance ( $p<0.05$ -0.001) (Figure 3 A, C), with AUC data showing decreases with Compound A and GSK137647 by 26% ( $p<0.05$ ) and 18% ( $p<0.05$ ) respectively (Figure 3 E, G). In combination with Sitagliptin, these compounds exhibited a further improvement on glucose excursion by 5-11%. The FFAR4 antagonist impaired the glucose lowering properties of Compound A and GSK137647 by 77% ( $p<0.05$ ) and 89% ( $p<0.05$ ) respectively (Figure 3 A, C).

These effects on blood glucose control were accompanied by relative changes in insulin secretion. Agonising FFAR4 with Compound A ( $p<0.05$ ) and GSK137647 ( $p<0.001$ ) increased plasma insulin by 20% and 54% when assessed with AUC data (Figure 3 F, H). Compound A in combination with Sitagliptin demonstrated an additive 12% insulinitropic effect compared to Compound A alone, whilst GSK137647 combinational therapy resulted in a 9% reduction compared to agonist alone (Figure 3 B, D). The FFAR4 antagonist AH-7614 inhibited the insulinitropic response of Compound A by 30% and GSK137647 by 76% ( $p<0.001$ ).

GSK137647 increased plasma GLP-1 ( $p<0.05$ ) and GIP ( $p<0.05$ ), whilst Compound A increased plasma GLP-1 ( $p<0.05$ ) but had no effect GIP secretion (Figure 4 A, B, D, E). GSK137647 ( $p<0.01$ ) and Compound A ( $p<0.01$ ) reduced DPP-IV activity, with activity further diminished when administered in combination with Sitagliptin ( $p<0.001$ ) (Figure 4 C, F).

In addition to oral treatment, Compound A and GSK137647 were administered also by intraperitoneal injection to lean mice (Figure 5 A). Compound A significantly improved glucose excursion ( $p<0.01$ ), with GSK137647 ( $p<0.05$ ) eliciting a similar response.

### **3.4 Acute effects of FFAR4 agonists on appetite suppression in lean Swiss TO mice**

Compound A induced satiety after 30 min with further effects observed over the 3 h time period ( $p<0.05$ - $p<0.001$ ) (Figure 5 B). Sitagliptin impaired the satiation effect of Compound A at 60 min. GSK137647 inhibited food intake after 30 min, with lasting effects throughout the experimental timeframe ( $p<0.01$ - $p<0.001$ ) (Figure 5 C). Combination of GSK137647 with Sitagliptin impaired the appetite suppressive effects of the agonist from 60 min to 180 min ( $p<0.05$ - $p<0.001$ ).

## **4 Discussion:**

Recent interest in long chain fatty acid receptors has intensified due to identification of their involvement in the maintenance of glucose homeostasis through GPCR signalling. FFAR1 (GPR40) [31], GPR55 [32, 33] and GPR119 [33, 34] have been previously reported to regulate islet function and hormone secretion. In particular, orally administered FFAR1 agonist TAK-875 (Fasiglifam) entered stage III clinical trials with promising anti-diabetic effects of equal

304 potency to the sulphonylurea glimepiride, whilst omitting the risk of hypoglycaemia [35, 36].  
305 However, signs of liver toxicity were observed towards the latter stages of the trial [37].

306 FFAR4 (GPR120) is a rhodopsin (class A) type receptor which has recently been shown to  
307 have anti-diabetic properties through the regulation of insulin and incretin secretion [1, 7, 8].  
308 Recent studies have identified the expression and involvement of FFAR4 in the pancreatic islet  
309 and intestinal tract, with expression also identified in adipose tissue, lungs and pro-  
310 inflammatory macrophages. FFAR4 shares 10% homology with FFAR1 and has high affinity  
311 for long chain fatty acids. However, many FFAR4 agonists can act as dual agonists with  
312 FFAR1, such as synthetic GW9508, therefore the identification of selective FFAR4 agonists is  
313 required to fully evaluate the role of the receptor in the maintenance of glucose homeostasis  
314 [7, 8].

315 In the present study, the therapeutic potential of two novel synthetic agonists (Compound A,  
316 GSK137647), which have been shown to exhibit selective properties towards FFAR4, were  
317 assessed [20-21]. Insulin secretion studies demonstrated that both agonists enhanced glucose  
318 stimulated insulin secretion from pancreatic BRIN-BD11 cells. Compound A and GSK13647  
319 displayed similar potencies at basal glucose levels ( $EC_{50}$  of  $\sim 10^{-7}$  mol/l). Stimulatory glucose  
320 concentrations, GSK137647 exhibited an  $EC_{50}$  of  $10^{-10}$  mol/l, compared with an  $EC_{50}$  of  $10^{-8}$   
321 mol/l for Compound A. Neither agonist imparted adverse effects on cell viability as assessed  
322 using MTT.

323 To demonstrate the selectivity of the novel agonists towards FFAR4, potent antagonists for  
324 FFAR4 and FFAR1 were employed. In the presence of FFAR1 antagonist (GW1100), the  
325 insulin secretory responses to both Compound A and GSK137647 was relatively unaffected.  
326 In contrast, the FFAR4 antagonist (AH-7614) impaired the insulinotropic properties of both  
327 agents, suggesting that the two agonists stimulate glucose dependent insulin secretion through

328 FFAR4 and not FFAR1 in the pancreatic beta cell. Numerous endogenous FFAR4 ligands have  
329 been shown to stimulate other fatty acids receptors, including FFAR1 [3, 8].

330 Immunohistochemistry demonstrated the highly abundant expression and co-localisation of  
331 FFAR4 and insulin in both the pancreas of lean and diabetic mice. Consistent with this, gene  
332 expression analysis demonstrated an upregulation of FFAR4 in BRIN-BD11 cells exposed to  
333 hyperglycaemia, suggesting that FFAR4 may have a regulatory role in islets exposed to  
334 diabetic stress factors. Interestingly, agonist treatment under normoglycaemic conditions  
335 significantly attenuated FFAR4 gene expression, with no effect demonstrated on FFAR4  
336 protein concentrations. The glucose responsive properties of the receptor expression promotes  
337 FFAR4 as a novel therapeutic target.

338 In HFF mice, both Compound A and GSK137647 demonstrated acute glucose lowering and  
339 insulintropic properties. Previously, FFAR4 activation was shown to augment glucagon  
340 release from pancreatic alpha cells [38]. Although glucagon opposes the biological actions of  
341 insulin, improved glucose tolerance is exhibited upon FFAR4 agonist treatment. This indicates  
342 superior secretory actions of FFAR4 agonism on insulin releasing beta cells. Interestingly,  
343 FFAR4 activation has also been shown to inhibit somatostatin release from pancreatic delta  
344 cells, which may have indirectly enhanced the insulintropic actions of FFAR4 agonists  
345 observed in this study [39]. Although numerous glucoregulatory hormones are released upon  
346 FFAR4 activation, the anti-inflammatory effects and potentiation of glucose uptake in  
347 peripheral tissues may have also attributed to the glucose lowering effects exhibited by FFAR4  
348 [9].

349 Conflicting results have been reported of the effect of FFAR4 activation of GLP-1 secretion  
350 [1, 16]. In the present study, GSK137647 was shown to induce GLP-1 and GIP secretion, whilst  
351 Compound A only stimulated GLP-1 secretion. To prolong the bioactivity of endogenously



released incretins, the DPP-IV inhibitor Sitagliptin was examined in combination with the FFAR4 agonists. Under these conditions, Compound A and GSK137647 exhibited enhanced glucose lowering capabilities, by stimulating incretin (GLP-1, GIP) and insulin secretion through FFAR4 activation, supplemented with prolonged incretin action through DPP-IV inhibition. DPP-IV inhibition however countered the inhibitory effects of both FFAR4 agonists on feeding activity. Further studies are required to fully understand the mechanism but inhibition of DPP-IV-mediated degradation of PYY(1-36) to the active form PYY(3-36) seems likely [40].

To establish that the glucose lowering properties of the agonists was not based solely on incretin secretion, each agonist was administered i.p. in combination with glucose. Both GSK137647 and Compound A improved glucose excursion confirming that FFAR4 activation also directly stimulates beta cell function. The selectivity of the novel agonists was tested using the FFAR4 antagonist AH-7614, which significantly impaired the insulinotropic and glucose lowering capabilities of GSK137647 and Compound A.

In conclusion, selective FFAR4 agonists enhance glucose stimulated insulin secretion in a concentration-dependent manner, whilst exhibiting no affinity for FFAR1. Expression analysis demonstrated glucose responsive properties of FFAR4 expression under hyperglycaemic stress, a novel finding which may aid the development of future anti-diabetic therapeutics. Activation of FFAR4 was associated with acute stimulatory effects on GLP-1 and GIP secretion. GSK137647 was the most potent agonist in terms of insulin and incretin secretion, suggesting that this agonist should be considered for further investigation. In addition, it has been shown for the first time that FFAR4 agonist combinational therapy with sitagliptin further improves glucose tolerance and may provide a novel approach for the treatment of type 2 diabetes.

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## 497 **Figure Legends**

498 **Figure 1.** Acute insulintropic effects of FFAR4 agonists (A, C) Compound A and (B, D)  
499 GSK137647 alone and in combination with FFAR4 antagonist AH-7614 ( $10^{-5}$  mol/l) or FFAR1  
500 antagonist GW1100 ( $10^{-5}$  mol/l) at 5.6 mM and 16.7 mM glucose in clonal pancreatic BRIN-  
501 BD11 cells. Alanine (10 mmol/l) was used as positive control. MTT cell viability analysis  
502 demonstrating cytotoxicity of (A, C) Compound A and (B, D) GSK137647 on BRIN-BD11  
503 cells. Hydrogen peroxide (1 mmol/l) was used as a positive control. Values are mean  $\pm$  SEM  
504 (n=8) for insulin secretion and (n=4) for cell viability. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001,  
505 compared to saline control. †p<0.05, ††p<0.01, †††p<0.001, compared to agonist alone.

506 **Figure 2.** Localisation of (A, B) 4', 6 diamidino-2-phenylindole (DAPI) nuclear stain, (C, D)  
507 FFAR4, (E, F) insulin and (G) double immunofluorescence of FFAR4 and insulin in high fat  
508 fed (A, C, E, G) and lean (B, D, F, H) pancreatic islets at X40 magnification. Examples of  
509 double immunofluorescence indicated by white arrows. qPCR and western blot analysis  
510 demonstrating the effect of FFAR4 agonist treatment on FFAR4 mRNA and protein  
511 concentrations at (I, K) 5.6 mM and (J, L) 16.7 mM in clonal pancreatic BRIN-BD11 cells  
512 after 4 h treatment. Expression was normalised to GAPDH for qPCR (n=3) and  $\beta$ -actin for  
513 western blotting (n=2; two independent experiments with two technical replicates). Values are  
514 presented as mean  $\pm$  SEM. \*p<0.05, \*\*p<0.01, compared to glucose control.

515 **Figure 3.** Acute effects of FFAR4 agonists Compound A and GSK137647 on plasma glucose  
516 (A, C), insulin (B, D) and respective AUC (E-H). Glucose (18 mmol/kg bw) was administered  
517 orally alone or in combination with FFAR4 agonist Compound A or GSK137647 (0.1  $\mu$ mol/kg  
518 bw) and either the FFAR4 antagonist AH-7614 (0.1  $\mu$ mol/kg bw) or Sitagliptin (50 mg/kg bw)  
519 to HFF mice (n = 6). Values are presented as mean  $\pm$  SEM. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001,

compared to HFF glucose control. †p<0.05, ††p<0.01, †††p<0.001, compared to agonist alone.

**Figure 4.** Acute effects of FFAR4 agonists Compound A and GSK137647 (0.1 µmol/kg bw) on circulating (A, D) total GLP-1, (B, E) total GIP and (C, F) DPP-IV activity. Glucose (18 mmol/kg bw) was administered orally alone or in combination with FFAR4 agonist to HFF mice (n = 6). Values are presented as mean ± SEM. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, compared to HFF glucose control.

**Figure 5.** Acute effects of i.p. administration of FFAR4 agonists Compound A and GSK137647 (0.1 µmol/kg bw) on (A) glucose tolerance and (B, C) cumulative food intake. Glucose (18 mmol/kg bw) was administered i.p. in combination with FFAR4 agonist to lean Swiss TO mice for glucose tolerance (n = 6). For satiety analysis, FFAR4 agonists were orally administered alone or in combination with DPP-IV inhibitor (Sitagliptin) to 18 h fasted lean Swiss TO mice. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, compared to saline control. †p<0.05, ††p<0.01, †††p<0.001, compared to agonist alone.

#### **Supplementary data**

**Figure 1.** Effects of Compound A and GSK137647, with half maximum effective concentration (EC<sub>50</sub>) values, on insulin release from clonal pancreatic BRIN-BD11 cells at (A) 5.6 mM and (B) 16.7 mM glucose concentrations. Results are the mean ± SEM (n=8).

**Figure 2.** Effect 150-day high fat fed diet on glucose tolerance and insulin secretion in 18 h fasted Swiss TO mice. Animals were subjected to an oral glucose tolerance test (18 mmol/kg bw) with (A, B) glucose tolerance, (C, D) insulin secretory response, (E) fasting plasma glucose



and (F) bodyweight determined. Results are the mean  $\pm$  SEM (n=6). \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 compared to lean control.

**Figure 3.** Effect 150-day high fat fed diet on glucose tolerance and insulin secretion in 18 h fasted Swiss TO mice. Animals were subjected to an insulin sensitivity test (40U/kg bodyweight; dissolved in 0.9% saline, i.p. injection). (A) Plasma glucose and (B) respective AOC are shown. Results are mean  $\pm$  SEM (n=8). \*\*p<0.01, compared to lean control.

563 **Figure 1**

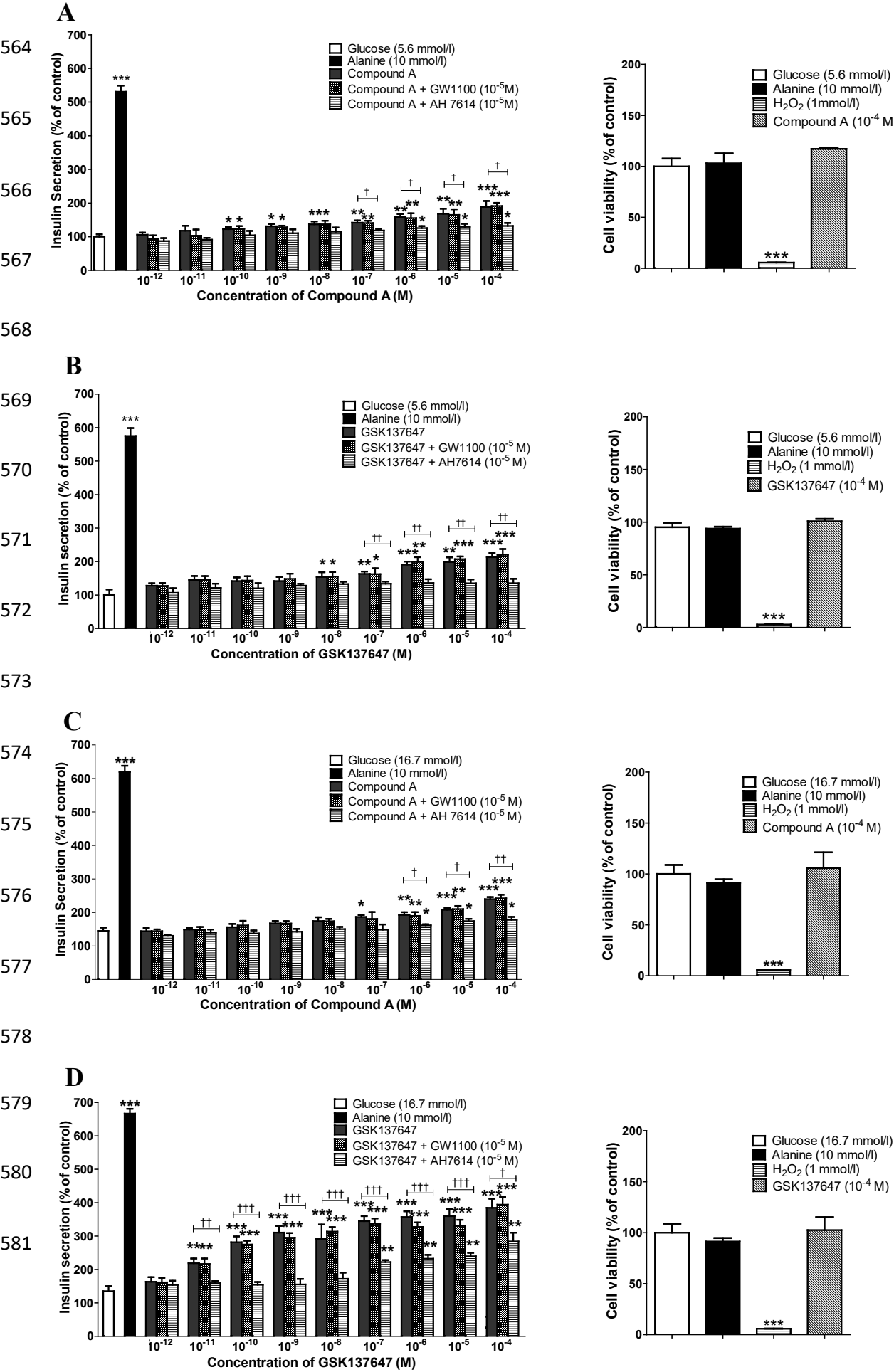


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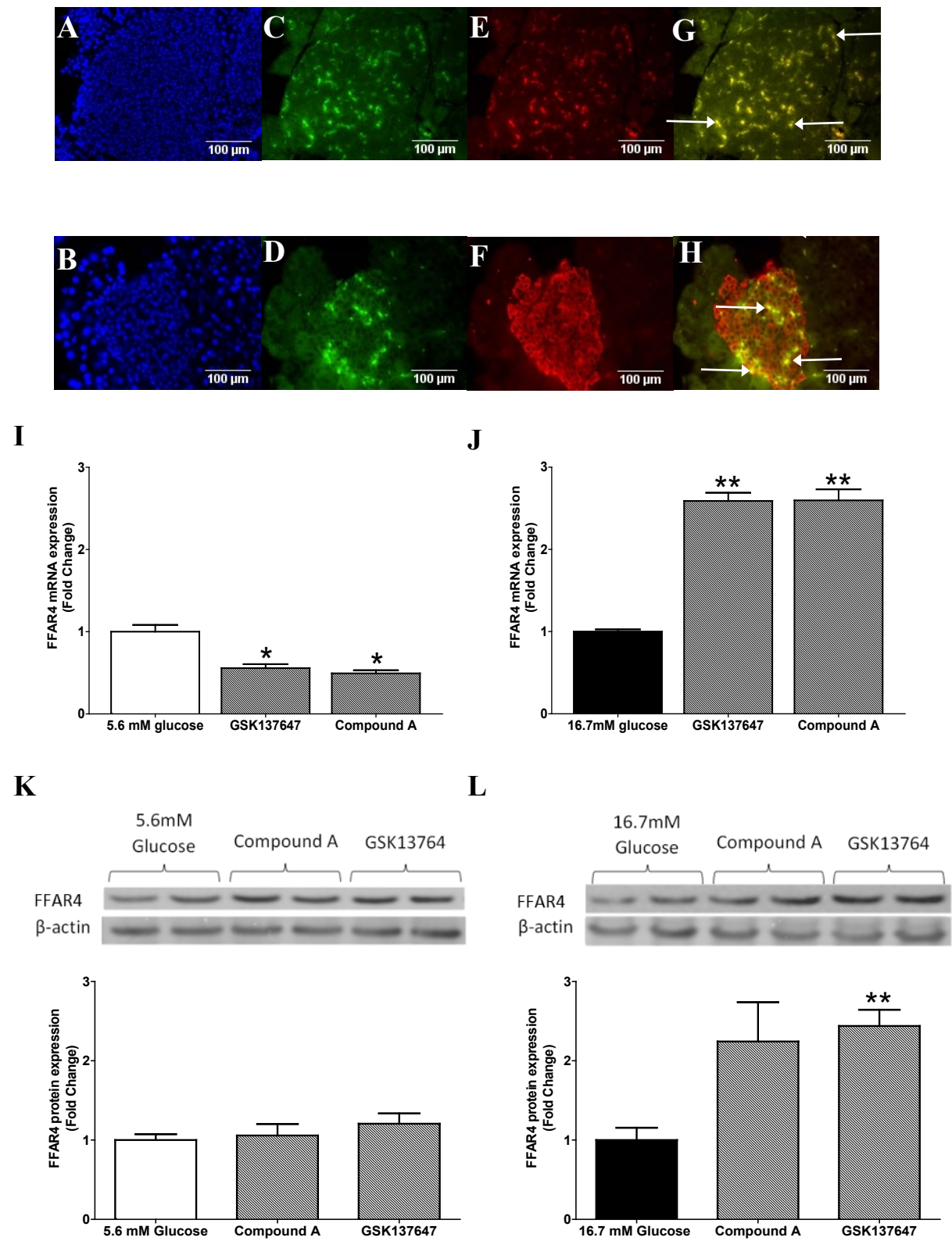


Figure 3.

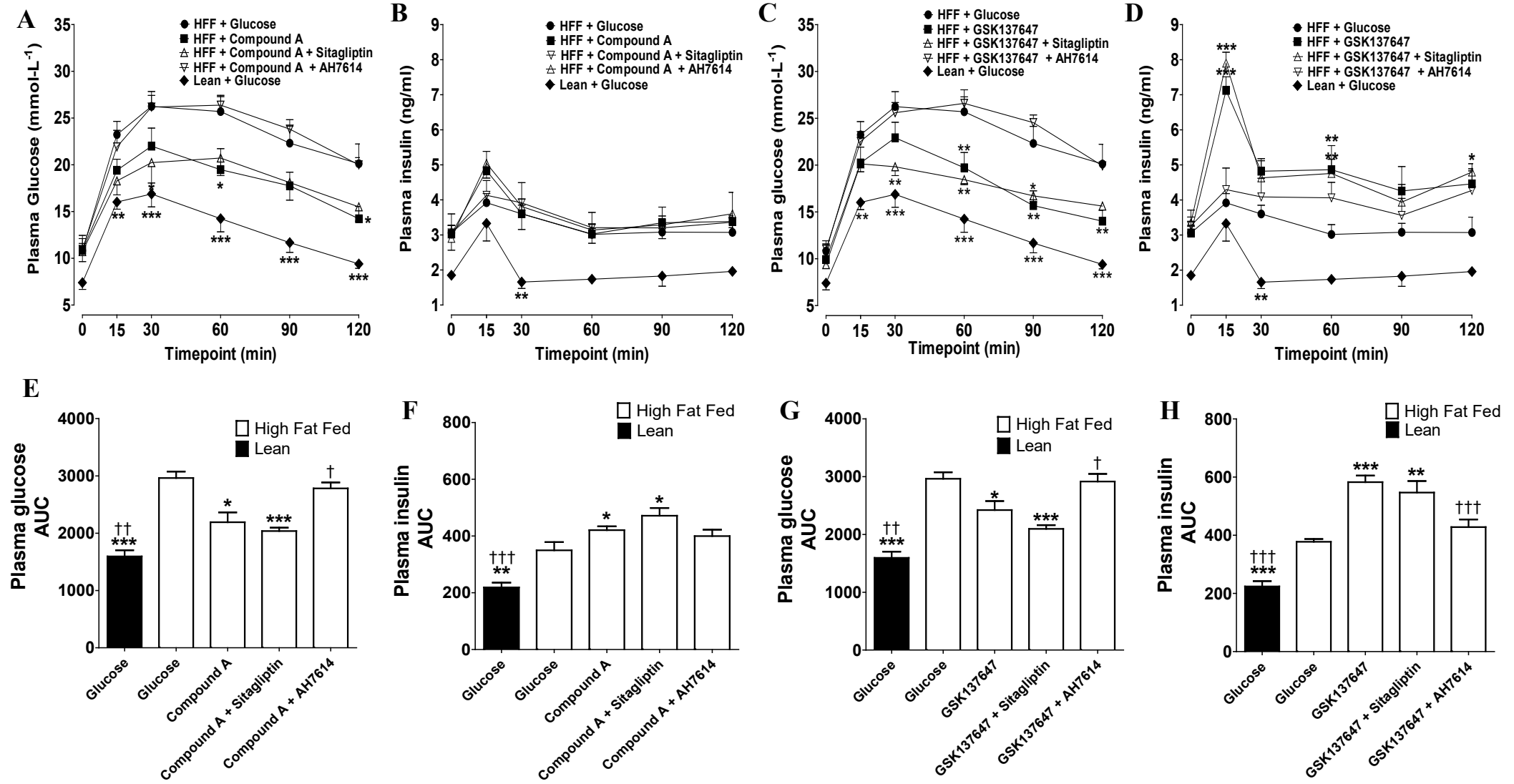
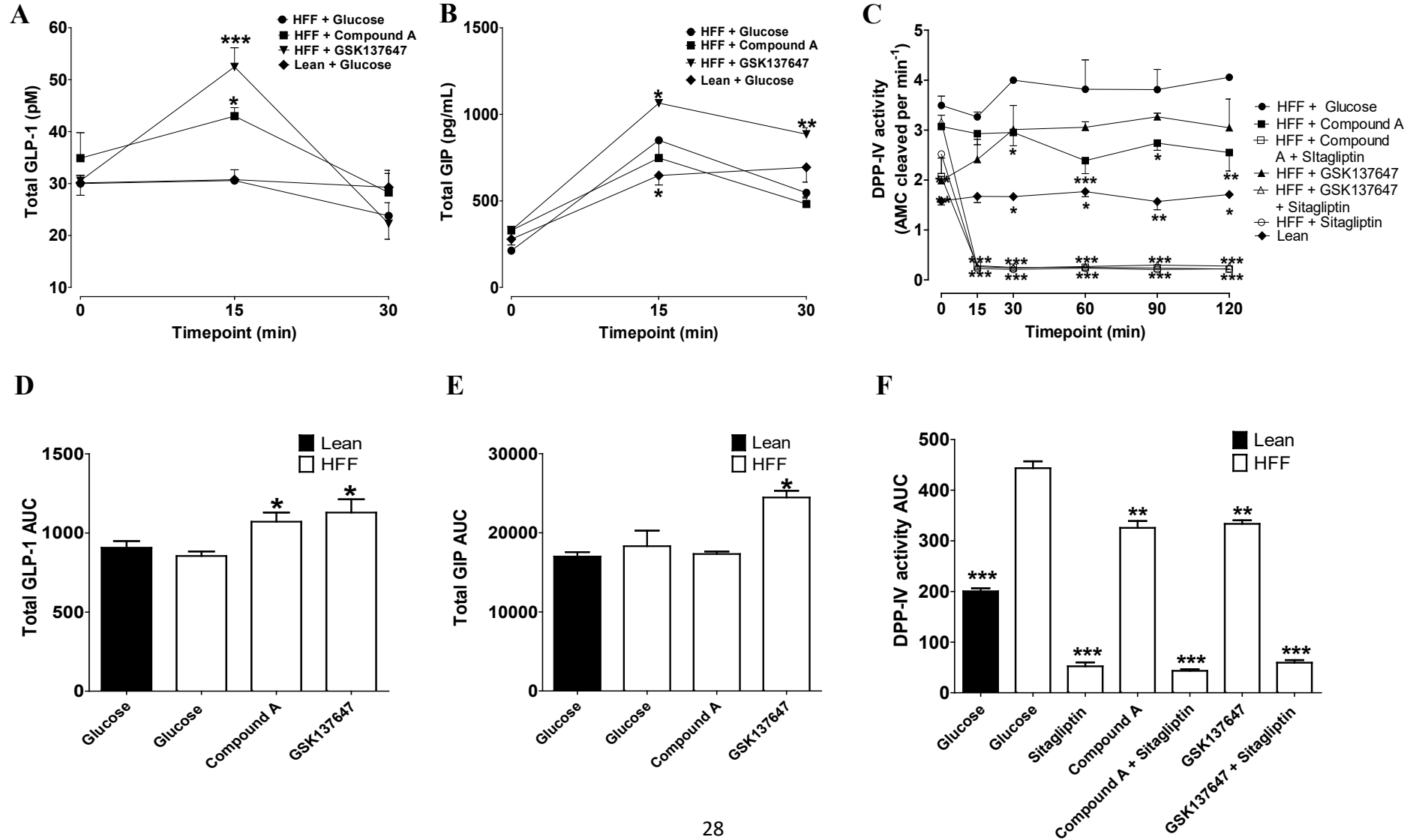
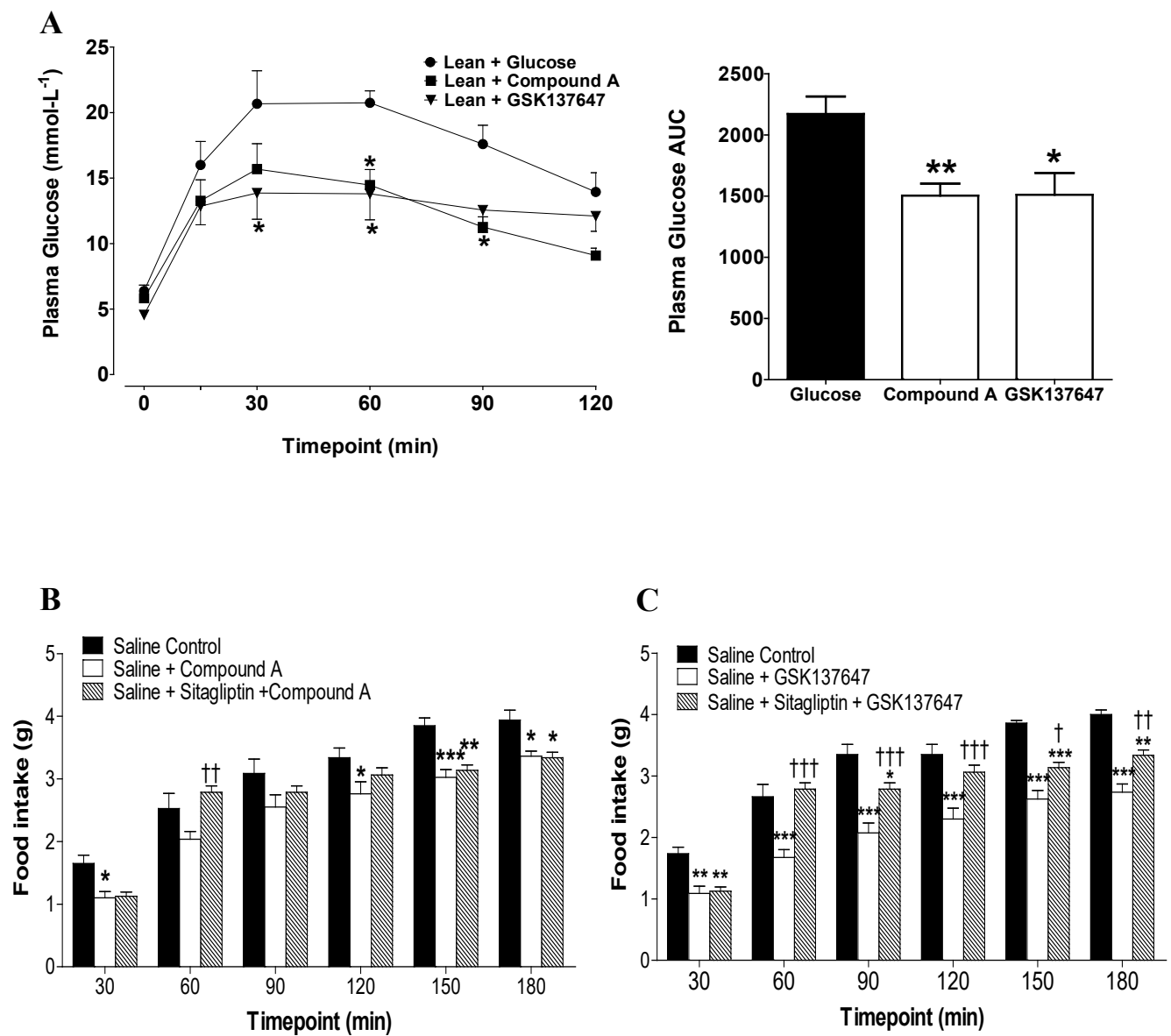


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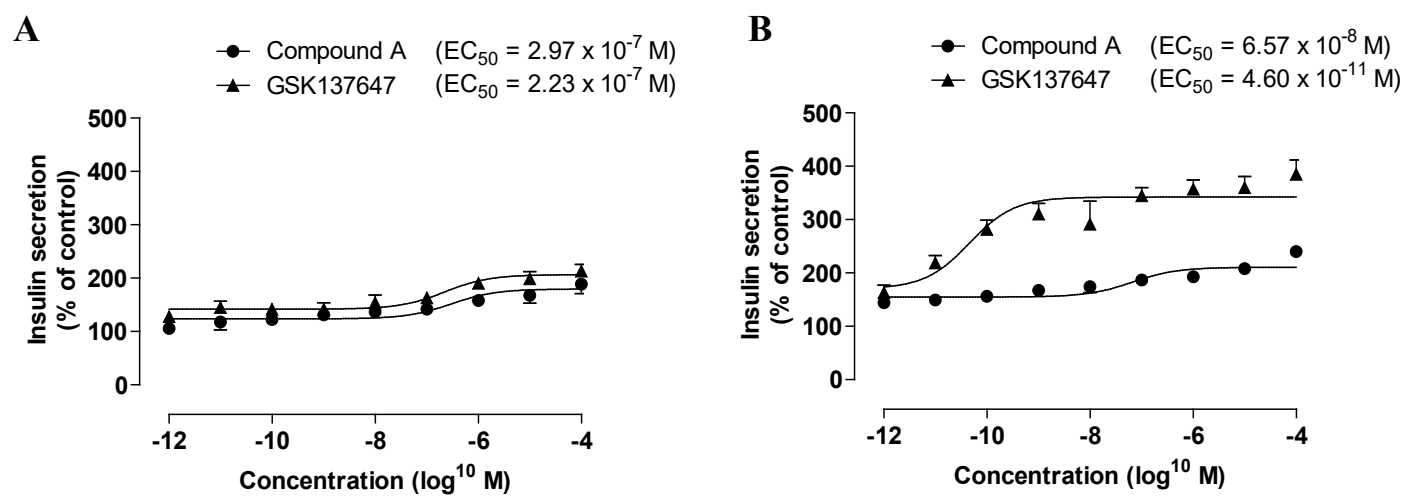


**Figure 5**



## Supplementary material

Figure 1



**Figure 2**

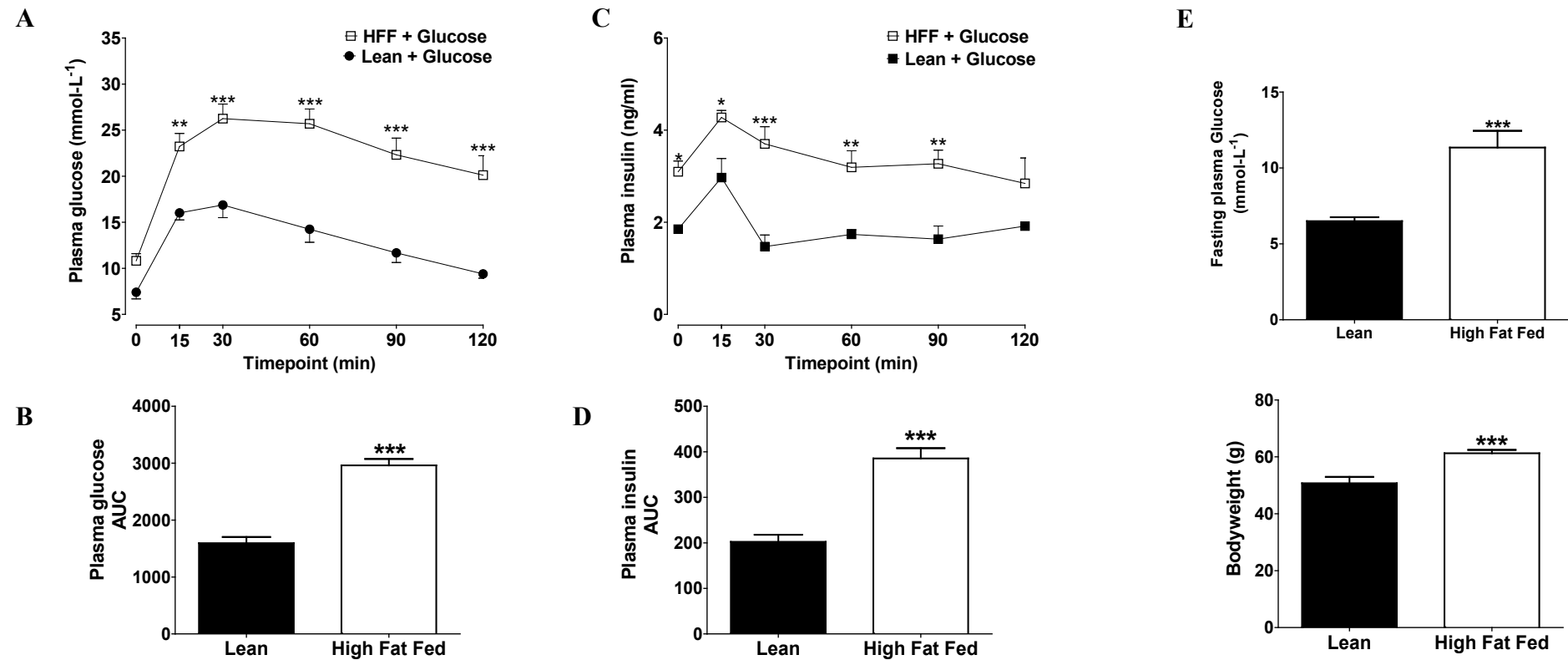




Figure 3

